The activation segment of procarboxypeptidase A from porcine pancreas constitutes a folded structural domain

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The controlled action of trypsin on porcine pancreatic procarboxypeptidase A releases a large activation peptide which contains the activation segment of the proenzyme. Circular dichroism studies indicate that the isolated activation peptide contains a high percentage of residues in ordered secondary structures (mainly α-helix). This result agrees with predictions of secondary structure carried out on the published amino acid sequence of the homologous rat proenzyme. Moreover, proton magnetic resonance spectroscopy shows that the peptide adopts a thermostable tertiary structure with characteristics typical of globular proteins. The results as a whole indicate that the activation segment of porcine pancreatic procarboxypeptidase A constitutes a folded structural domain.

Procarboxypeptidase A *Activation segment* *Conformation* *Structural domain* *Circular dichroism* *Nuclear magnetic resonance*

1. INTRODUCTION

The activation of different pancreatic procarboxypeptidases A and B by the action of trypsin proceeds through the release and degradation of an N-terminal polypeptidic region of ~100 residues [1–3]. The functional role of this activation region in the proenzyme is known to be related to the maintenance of the enzymic activity at low level [3,4]. However, neither the conformation of this large activation segment nor its mode of operation is known yet.

We have isolated a large peptide from porcine pancreatic procarboxypeptidases A by limited proteolysis with trypsin [3]. By amino acid and N-terminal analysis it was shown that this peptide contains the whole activation segment of the proenzyme. Furthermore, the peptide is a specific competitive inhibitor of pancreatic carboxypeptidases A with a K_i in the nM range. Here a conformational analysis of the isolated peptide by circular dichroism and by proton magnetic resonance spectroscopy is reported. The results indicate that in aqueous solution the peptide folds to form a high percentage of α-helix and a low percentage of β-form. Moreover, the folded peptide displays several characteristics typical of the tertiary structure of globular proteins. The results as a whole indicate that the activation segment of porcine procarboxypeptidase A constitutes a folded structural domain. This is the first time that data on the conformation of the activation segment of a pancreatic procarboxypeptidase are presented.

Abbreviations: CD, circular dichroism; NMR, nuclear magnetic resonance, RCS, ring-current shifted

2. MATERIALS AND METHODS

Monomeric procarboxypeptidase A was isolated from the acetone powder of porcine pancreas as in [2]. The activation peptide of monomeric procarboxypeptidase A was obtained by limited proteolysis of this zymogen with trypsin followed by

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chromatography on DEAE–Sepharose in the presence of 7 M urea as in [3]. After urea removal, the activation peptide was precipitated with acetone, recovered by centrifugation, dried under vacuum and stored as a powder at −20°C

Circular dichroism studies were performed in a Roussel-Jouan Dichrograph II, at 25°C, under constant nitrogen flush. The concentration of the peptide studies was estimated by UV spectrophotometry at 278 nm [3] and by a Lowry–deoxycholate colorimetric assay [5] using procarboxypeptidase A as standard. A mean residual M_r of 115 for the acitvation peptide was used in the numerical calculations [3].

Proton nuclear magenetic resonance spectra were recorded at 270 MHz in a Bruker WH270 instrument, equipped with an Oxford Instruments 6.4 T superconducting magnet. Samples were dissolved in 99.8% ^2H_2O at ~5 mg/ml. The salt concentration was raised by direct addition of concentrated NaCl in ^2H_2O,and the p^{2}H adjusted by addition of small aliquots of NaO^{2}H of ^{2}HCl solutions. Chemical shifts were referenced to the sharp resonance of small traces of acetone in the sample to which we have assigned a shift of 2.240 ppm.

3. RESULTS AND DISCUSSION

Electrophoretic analysis of the products generated during digestion of monomeric procarboxypeptidase A from procine pancreas by low concentrations of trypsin indicates (not shown) the appearance of two single protein species in a broad period of hydrolysis: carboxypeptidase A (M_r ~ 34000) and a large activation peptide (M_r ~ 11500) that contains the whole activation segment of the proenzyme [3]. This result suggests that the activation segment of the proenzyme adopts a folded conformation that hinders its attack by trypsin.

Spectroscopic analysis of the secondary structure of the isolated activation peptide in 0.1 M KCl (pH 7.4) by circular dichroism (fig. 1) confirms the ability of this protein to fold. Thus the appearance of a prominent ellipticity minimum at 220–222 nm, $[\theta]_{222} = 16400$ deg.cm^2.dmol^{-1}, followed by another clear minimum at 209 nm indicates a high content of α-helix for the peptide. On the basis of $[\theta]_{222} = -2340$ for the random coil and −30300 for the α-helix [6], the above experimental value at

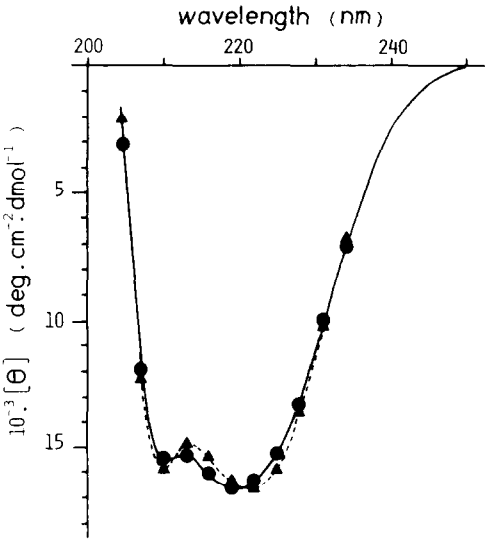

Fig. 1. Circular dichroism spectra of the activation peptide from porcine pancreatic procarboxypeptidase A. (—●—) experimental spectrum in 0.1 M KCl (pH 7.4), at 25°C; (—▲—) curve-fitted spectrum according to [6].

222 nm represents 46.5% helicity. Similar results were obtained when the salt concentration of the medium was varied from 10 mM to 0.5 M in KCl or in $CaCl_2$, the pH being kept at 7.4. A more precise analysis of the CD spectra has also been undertaken by application of different curve-fitting methods [6–8] from 234–204 nm. A reasonable fit was obtained following the method in [6] (fig. 1). From curve-fitting an α-helix content between 57–65%, and a β-structure content between 8–24%, was estimated for the different samples analysed. It should be noted that the estimation of the β-structure content by circular dichroism is usually subject to an appreciable level of uncertainty [9,10].

Comparison of the above results with the prediction of the secondary structure of the isolated peptide based on the amino acid sequence is hindered by the lack of sequence information. However, this analysis can be carried out on the amino acid sequence of the activation region of rat pancreatic procarboxypeptidase A in [11]. The high homology between the activation region of this proenzyme and other mammalian procarboxypeptidases shown by sequence and amino acid composition studies [3,11], justifies the proposed comparative

procedure. Application of the Chou and Fasman [12] predictive method (including boundary analysis) indicates a high probability of α-helix between residues 14–34 ($P_\alpha = 1.23$) and 71–93 ($P_\alpha = 1.22$) of the activation region of rat procarboxypeptidase A. Moreover, two other pieces of α-helix can also be predicted between residues 39–43 ($P_\alpha = 1.25$) and 57–64 ($P_\alpha = 1.17$), although there are some doubts about the existence of the former one. However, two stretches of β-conformation are predicted between residues 8–12 ($P_\beta = 1.33$) and 47–51 ($P_\beta = 1.29$). Therefore, the 94 residues of the activation region of rat procarboxypeptidase A could have between 55–61% of α-helix and around 11% of β-structure. These predictive figures fit fairly well with our estimation of secondary structure of the isolated activation segment of porcine procarboxypeptidase A by circular dichroism.

Proton magnetic resonance spectroscopy at 270 MHz indicates that the isolated activation peptide in 0.1 M NaCl (p^2H 7.8) is in a folded conformation and displays several characteristics of globular proteins. Thus several ring-current shifted resonances (probably from alkyl groups of apolar residues adjacent to aromatic rings) are apparent between 0–1 ppm, as shown in fig. 2A. The chemical shift of these resonances is sensitive to increase in temperature and completely disappears at 90°C (fig. 2B). A concomitant increase in intensity in the large peak at ~0.9 ppm (attributed mainly to alkyl groups) is also observed during this thermal perturbation. Both phenomena are a consequence of the unfolding of the protein [13,14]. Some of the above ring-current shifted resonances at high field are also sensitive to the changes in p^2H, specially in the range 6.0 to 8.0 (not shown). However, the low field region of the spectrum,

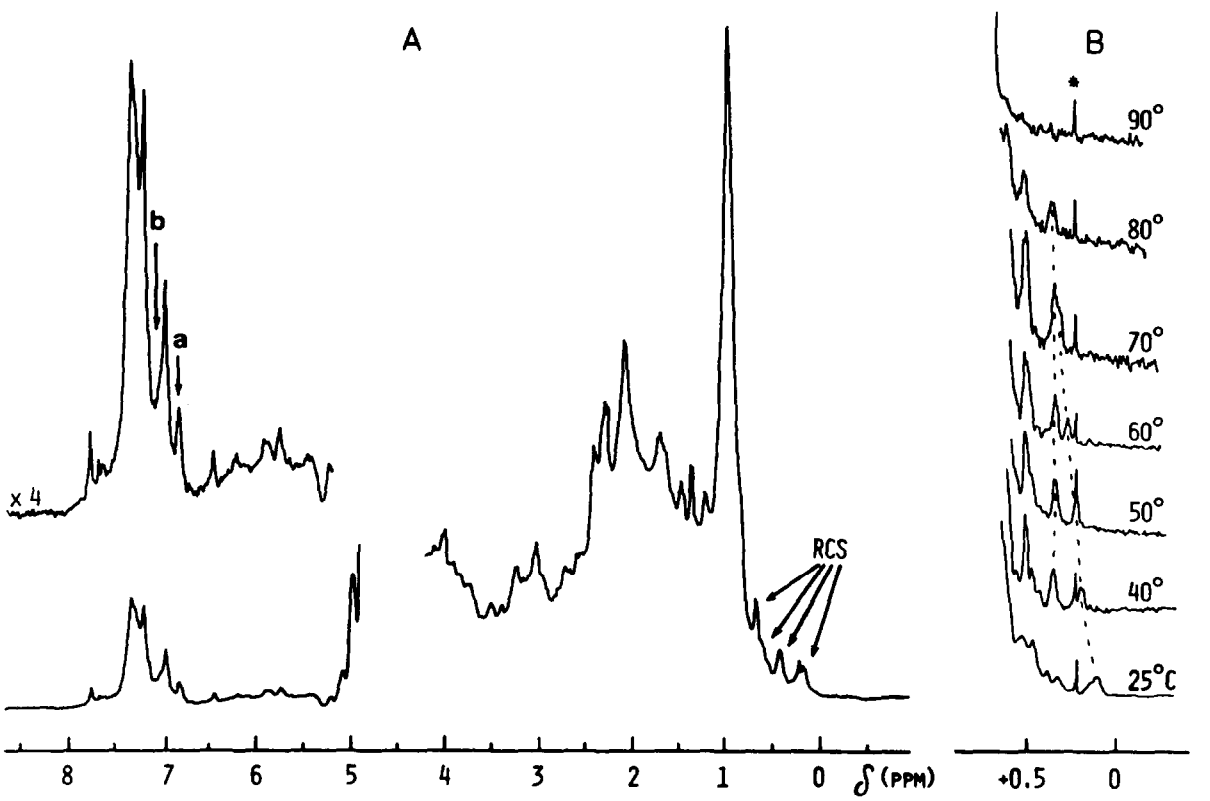

Fig. 2. Proton magnetic resonance spectra at 270 MHz of the activation peptide from porcine procarboxypeptidase A: (A) full spectrum in 0.1 M NaCl (p^2H 7.8), at 40°C; (B) effect of thermal denaturation on the ring-current shifted (RCS) resonances at upfield (resolution enhanced by convolution difference). The resonance at 0.23 ppm (*) is from an organic contaminant.

largely dominated by the resonance from phenylalanines, is also dependent on the temperature. When the temperature is increased from 25–90°C, the resonance at 6.87 ppm (probably from a tyrosine ring and denoted by (a)) decreases in intensity and a new resonance appears at 7.08 ppm (denoted by (b)). Moreover, the broad resonances located between 5.25–6.5 ppm (probably from α-carbon protons) disappear during this increase in temperature (not shown). It should be stressed that a high stability is shown by the protein segment against thermal denaturation in spite of its lack of disulfide bridges [3]. Its high content of apolar and aromatic residues could be an explanation for this behaviour. The above observations demonstrate that the activation segment is in a compact and folded conformation in aqueous solution.

What is the conformation of the activation region within procarboxypeptidase A? No direct analyses are available yet, however the high stability shown by the conformation of the isolated activation segment against some environmental changes (temperature, ionic strength), and the relative resistance to degradation whilst trypsin severs it cleanly from the proenzyme, suggest that in the proenzyme the activation segment keeps a folded conformation and constitutes a folded domain. The study of both the conformation of the activation segment and its spatial location within procarboxypeptidase A could help the elucidation of the detailed mechanism of action and inhibition of the active enzyme, as discussed in [3].

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